

ALKYLATION OF REDUCED  $\kappa$ -CASEIN

The study of polymorphism of  $\kappa$ -casein by the technique of gel electrophoresis requires the reduction of disulfide bonds. This is necessary because native  $\kappa$ -casein does not yield discrete bands after starch- or polyacrylamide-gel electrophoresis, whereas reduction of the protein yields material which produces discrete bands. In this way, differences in the electrophoretic distributions of components of reduced  $\kappa$ -casein prepared from milks of individual cows have been detected and ascribed to polymorphism (3, 6, 7).

Further investigation of reduced  $\kappa$ -casein requires the preparation of the proteins in a stable reduced form. Several alkylating agents can be utilized to stabilize the thiol groups after reduction of  $\kappa$ -casein. Implicit in these studies is the requirement that the reduction-alkylation procedure be complete for disulfide (cystine) or thiol (cysteine) groups, or both, and that the method does not lead to alkylation of other sites in the polypeptide chain. A method is presented for the complete reduction and alkylation of  $\kappa$ -casein utilizing iodoacetamide (IAA-NH<sub>2</sub>) which, under the prescribed conditions, does not result in any detectable nonspecific alkylation. A comparison was made between the amino acid composition of the iodoacetamide-treated  $\kappa$ -casein with that of  $\kappa$ -casein alkylated with acrylonitrile (AN) (8).

$\kappa$ -Casein was isolated from acid-precipitated casein obtained from pooled skim milk by the urea-H<sub>2</sub>SO<sub>4</sub> method of Zittle and Custer (9). The protein was purified by precipitation from an alcoholic solution according to McKenzie and Wake (2). The twice-precipitated material showed no other casein components after polyacrylamide-gel electrophoresis. S-cyanoethyl- $\kappa$ -casein was prepared by addition of a tenfold excess of  $\beta$ -mercaptoethanol ( $\beta$ -ME) (based on ten moles of -SH per 100,000 g protein) to a 1% solution of  $\kappa$ -casein in 8 M urea-pH 8.0 veronal buffer (0.1 M). The reduction was carried out for 1 hr, after which a three-molar excess of AN (based on total -SH content) was added. The pH was maintained at 8.0 by addition of 0.1 N NaOH, then lowered to 5.0 after 15 min. The reaction mixture was exhaustively dialyzed against distilled water and finally lyophilized. Previous studies (8) indicated that alkylation with IAA-NH<sub>2</sub> under the above conditions [8 M urea-pH 8.0 veronal (0.1 M)] resulted in retardation in electrophoretic mobilities, suggesting nonspecificity of alkylation. No alterations in electrophoretic mobilities were observed following alkylation, according to the procedure described below. S-carboxamido-methyl- $\kappa$ -casein was prepared by addition of a tenfold excess of  $\beta$ -ME (calculated as above) to a 1% solution of  $\kappa$ -casein in 4 M urea-pH 7.0 imidazole buffer (0.01 M). The reduced protein

was alkylated with a three-molar excess of IAA-NH<sub>2</sub> for 10 min, while maintaining the pH at 7.0. Residual IAA-NH<sub>2</sub> was reacted with an excess of  $\beta$ -ME before dialysis against distilled water and lyophilization.

Each protein was analyzed for its complete amino acid composition by the method of Piez and Morris (4) and the data compared with native  $\kappa$ -casein. About 2.0 mg of protein was hydrolyzed with glass-distilled 6 N HCl in a sealed, evacuated tube. Hydrolysis for each protein were carried out in triplicate at 110 degrees in a circulating-air laboratory oven. Data reported in Table 1 were calculated by finding molar ratios based on eight different amino acids (Asp, Pro, Ala, Met, Leu, Phe, His, and Arg). The molar ratios so obtained were multiplied by whole-number factors so that the ratios derived from each of the eight amino acids were raised to the same numerical level. Calculations for the three proteins were carried out in an identical manner.

An inspection of the table reveals that for native  $\kappa$ -casein, 1.6 residues of  $\frac{1}{2}$  Cys were found. This is an uncorrected value which does not take into account any destruction during hydrolysis. However, for S-cyanoethyl- $\kappa$ -casein and S-carboxamido-methyl- $\kappa$ -casein, the  $\frac{1}{2}$  Cys values, calculated as S-carboxyethyl-cysteine (S-CEC) in the former case and S-carboxymethyl-cysteine (S-CMC) in the latter case, are very good approximations to the value 2.0. This finding demonstrates that for each alkylating agent the thiol groups are completely alkylated. However, alkylation with AN resulted in a reduction of about 45% of the Lys residues. This loss can probably be accounted for by the appearance of a new peak in the elution patterns immediately following Phe (1). This peak is presumed to be related to the alkylation of lysine residues. A second minor peak also appears immediately preceding Ileu and remains unidentified. In the alkylation with IAA-NH<sub>2</sub>, there was a Lys decrease of only 7%, which represents less than one full residue. No new peaks of any consequence appeared in the elution patterns of hydrolysates of S-carboxamido-methyl- $\kappa$ -casein. All other amino acids for the three proteins in the table appear to have approximately equivalent numbers of residues. Deviations from whole-number values can be attributed to heterogeneity of the pooled  $\kappa$ -casein, and the apparent differences in Thr and Ser to differences in the rate of destruction of these amino acids in the three proteins.

From the present data nothing can be inferred about alkylation of sites resulting in bonds labile under the conditions of hydrolysis and chromatography employed. In addition, alkylation of carbohydrate moieties of  $\kappa$ -casein would not be revealed unless the resulting com-

TABLE 1  
Amino acid composition of kappa-casein, S-cyanoethyl- and S-carboxamido-methyl-kappa-casein <sup>a</sup>

Amino acids	$\kappa$ -Casein		S-Cyanoethyl- $\kappa$ -casein		S-Carboxamido-methyl- $\kappa$ -casein	
	$\pm .05^s \bar{X}^c$		$\pm .05^s \bar{X}^c$		$\pm .05^s \bar{X}^c$	
Asp	11.7	.22	12.0	.23	12.4	.29
Thr <sup>b</sup>	11.2	.21	11.6	.24	12.6	.34
Ser <sup>b</sup>	10.6	.20	11.0	.23	11.6	.31
Glu	25.5	.47	26.2	.55	26.4	.70
Pro	17.2	.38	17.5	.42	17.9	.53
Gly	2.7	.053	2.7	.18	2.8	.077
Ala	12.5	.26	12.8	.32	13.3	.41
$\frac{1}{2}$ Cys <sup>b</sup>	1.6	.037	0	.	0	.
Val	9.7	.18	9.8	.22	10.5	.28
Met	1.9	.048	1.9	.039	1.8	.046
Ileu	10.5	.20	11.4	.32	11.0	.29
Leu	8.8	.19	8.7	.20	8.9	.27
Tyr <sup>b</sup>	8.5	.16	8.6	.20	8.4	.28
Phe	4.2	.084	4.2	.095	4.1	.13
Lys	10.0	.22	5.6	.14	9.3	.25
His	2.9	.071	2.9	.090	2.9	.091
Arg	5.2	.11	5.0	.15	4.7	.14
S-CEC	....	....	2.1	.12	....	....
S-CMC	....	....	....	....	1.8	.050

<sup>a</sup> Values based on molar ratios (see text); each protein hydrolyzed in triplicate for 24 hr at 110 degrees in sealed, evacuated tubes.

<sup>b</sup> Uncorrected for destruction.

<sup>c</sup> Measure of repeatability at 95% confidence limit.

pounds survived hydrolysis, chromatography, and were ninhydrin-positive. A further point of interest is the finding of Plummer and Hirs (5), that ribonuclease B, a glycoprotein, is specifically alkylated by AN only at the thiol groups under their specified conditions, but IAA and its derivatives were nonspecific. This seems to be the inverse of the situation described for  $\kappa$ -casein. It should also be noted that the alkylation by AN of the whey proteins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, has also been shown to be nonspecific (1).

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#### REFERENCES

- (1) KALAN, E. B., NEISTADT, A., AND WEIL, L. 1965. Cyanoethylation of Milk Proteins and the Determination of S-Carboxyethylcysteine. *Fed. Proc.*, 24: 225.
- (2) MCKENZIE, H. A., AND WAKE, R. G. 1961. An Improved Method for the Isolation of  $\kappa$ -Casein. *Biochim. et Biophys. Acta*, 47: 240.
- (3) NEELIN, J. M. 1964. Variants of  $\kappa$ -Casein Revealed by Improved Starch Gel Electrophoresis. *J. Dairy Sci.*, 47: 506.
- (4) PIEZ, K. A., AND MORRIS, L. 1960. A Modified Procedure for the Automatic Analysis of Amino Acids. *Anal. Biochim.*, 1: 187.
- (5) PLUMMER, T. H., JR., AND HIRS, C. H. W. 1964. On the Structure of Bovine Pancreatic Ribonuclease B. Isolation of a Glycopeptide. *J. Biol. Chem.*, 239: 2530.
- (6) SCHMIDT, D. G. 1964. Starch-Gel Electrophoresis of  $\kappa$ -Casein. *Biochim. et Biophys. Acta*, 90: 411.
- (7) WOYCHIK, J. H. 1964. Polymorphism in  $\kappa$ -Casein of Cow's Milk. *Biochim. Biophys. Research Comm.*, 16: 267.
- (8) WOYCHIK, J. H. 1965. Preparation and Properties of Reduced  $\kappa$ -Casein. *Arch. Biochem. Biophys.*, 109: 542.
- (9) ZITTLE, C. A., AND CUSTER, J. H. 1963. Purification and Some of the Properties of  $\alpha_s$ -Casein and  $\kappa$ -Casein. *J. Dairy Sci.*, 46: 1183.